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Establishment and Characterization of Immortalized Gingival Epithelial and Fibroblastic Cell Lines for the Development of Organotypic Cultures

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Key Words

Gingival epithelium · Gingival fibroblasts · Human papillomavirus type 16 · Organotypic tissue

Abstract

In vitro studies using 3D co-cultures of gingival cells can resemble their in vivo counterparts much better than 2D models that typically only utilize monolayer cultures with short-living primary cells. However, the use of 3D gingival models is still limited through lack of appropriate cell lines. We aimed to establish immortalized cell line models of primary human gingival epithelium keratinocytes (HGEK) and gingival fibroblasts (GFB). Immortalized cell lines (HGEK-16 and GFB-16) were induced by E6 and E7 oncoproteins of human papillomavirus. In addition, 3D multilayered organotypic cultures were formed by embedding GFB-16 cells within a collagen (Col) matrix and seeding of HGEK-16 cells on the upper surfaces. Cell growth was analyzed in both immortalized cell lines and their parental primary cells. The expression levels of cell type-specific markers, i.e. cytokeratin (CK) 10, CK13, CK16, CK18, CK19 for HGEK-16 and Col I and Col II for GFB-16, were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Expansion of the primary cultures was impeded at early passages, while the transformed immortalized cell lines could be expanded for more than 30 passages.

In 3D cultures, immortalized HGEK formed a multilayer of epithelial cells. qRT-PCR showed that cell-specific marker expression in the 3D cultures was qualitatively and quantitatively closer to that in human gingival tissue than to monolayer cultures. These results indicate that immortalized gingival fibroblastic and epithelial cell lines can successfully form organotypic multilayered cultures and, therefore, may be useful tools for studying gingival tissue in vitro.

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Introduction

Gingival tissue comprises the superficial oral epithelium and underlying connective tissue. These tissues are the first locations to be affected by biofilms as well as the initiation sites for inflammatory processes. They are therefore recognized as the places for the initiation of periodontal diseases, a widespread group of destructive oral infection-driven inflammatory diseases that affect 48.2% of the USA population aged ≥30 years [Albandar, 2011].

Based on structural and functional differences, gingival epithelial tissue has been classified as junctional epithelium, oral sulcular epithelium and oral gingival epithelium. Oral junctional epithelium connects the tooth to the sub-

epithelial connective tissue and sulcular epithelium lines the lateral wall of the gingival sulcus. Besides its supporting functions, the permeability of the epithelium allows emigration of granulocytes to the biofilm surface but also enables microorganisms to migrate from the biofilm [Sandro et al., 1994], which is believed to play an important role during the process of periodontal infection. In the clinic, the appearance of a periodontal pocket, an abnormal depth of the gingival sulcus, is considered to be an indicator for periodontal infection. Interestingly, disappearance of the junctional epithelium and ulceration of sulcular epithelium during the formation of the periodontal pocket has been observed [Nanci and Bosshardt, 2006], which means that gingiva is not able to form tight connections after the disease. Therefore, it is of importance to understand how healthy non-keratinized sulcular and junctional epithelium is breached by the oral biofilms; what changes occur to these structures during disease processes, and if the molecular mechanisms of the pristine status recover after clinical improvements [Belibasakis et al., 2011]. In progressive periodontal diseases presenting with ulcerated epithelium, the gingival connective tissue (mainly fibroblasts) is directly exposed to the bacterial biofilm. As connective tissue also influences the formation of gingival epithelia [Karring et al., 1975; Smola et al., 1998], concomitant studies using both epithelial and connective tissue are crucial for understanding the pathogenesis of periodontal diseases [MacNeil, 2007].

In vitro studies on gingival tissue have mainly employed monolayer cultures. Due to the lack of normal differentiation, gingival cells grown under these conditions are deficient in differentiation-dependent cell polarization and cell-cell contacts [Radyuk et al., 2003]. Thus, organotypic cell culture models that mimic the morphological and functional features of their in vivo counterparts are highly anticipated despite their methodological complexity. One of the main methodological problems of establishing such 3D cultures is that primary cells have short life spans, as they only replicate for a few passages and therefore do not provide sufficient cell numbers for further use in organotypic cultures [Sacks, 1996; Pi et al., 2007]. To overcome this problem, previous work has made use of the E6/E7 oncoproteins from human papillomavirus type 16 (HPV16-E6/E7) to immortalize epithelial cells [Yeh et al., 2013].

In the present study, we used primary human gingival epithelial keratinocytes (HGEK) and human gingival fibroblasts (GFB). Our primary aim was to immortalize them through the expression of oncogene HPV16-E6/E7. In addition, we aimed to establish a standardized organo-

typic gingival tissue model mimicking an oral junctional epithelial interface in vitro, using both immortalized gingival epithelial cells and gingival connective tissue. The characteristics of the established epithelia and fibroblasts in a 2D monolayer and organotypic culture were determined by histology and cell differentiation through quantification of cytokeratin (CK) and collagen (Col) expression using quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and Methods

Primary Cultures

GFBs were collected from clinically healthy gingiva as previously described [Belibasakis et al., 2005] and HGEK were extracted following a published protocol [Guggenheim et al., 2009]. Ethical approval was granted from the Ethical Committee of the Dental Institute Board (StV Nr. 08/14). The study was conducted according to the guidelines of the Declaration of Helsinki. Written and informed consent was obtained from each patient before enrolment in the study. HGEKs were cultivated and passaged in defined keratinocyte serum-free medium (Gibco), supplemented with 100 U/ml penicillin (Sigma, St. Louis, Mo., USA), 100 µg/ml streptomycin (Sigma), 2 mM L-glutamine (Sigma) and 0.25 µg/ml Fungizone (Sigma). GFBs were cultivated and passaged in Dulbecco's modified Eagle's medium (DMEM):nutrient mixture F-12 (DMEM/F-12) media (Sigma) supplemented with 1.2 g/ml of sodium bicarbonate (Sigma), 10% FCS (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 0.05 µg/ml Fungizone. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 2 days. When the primary cell cultures reached confluence, cells were dissociated with trypsin/EDTA solution (Gibco, Life Technologies) just before transferring for experiments. Cell numbers were determined under light microscopy using a hemocytometer. Population doubling was calculated as log₂ (cell number at subculture/cell number plated) for growth kinetics determination.

Immortalization and Maintenance of Cultures

For immortalization, gingival cells were retrovirally transduced with virus coding either for pLXSN empty vector control (pLXSN) or HPV16-E6E7 (pLXSN-HPV16-E6E7) as described previously [Akgul et al., 2010]. Briefly, primary cells were seeded at a cell density of 2×10^5 cells/cm² in 6-cm dishes. Retroviral supernatants were mixed with an equal volume of DMEM in the presence of 5 µg/ml of hexadimethrine bromide (Polybrene; Sigma) and added to the keratinocytes. Spin infection was made by centrifugation at 300 g for 1 h; the cells were then washed with PBS and cultured in corresponding fresh medium. After 2 days, cells were selected with G418 (Sigma-Aldrich; 100 µg/ml for keratinocytes and 500 µg/ml for fibroblasts) for 7 days after which only infected cells survived. The use of pooled stable cell populations minimizes possible variations due to randomness of the viral integration site in the cellular chromosomes. Established HGEK-16 or GFB-16 cells were seeded at 3,333 cells/cm² and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for passaging. HGEK-16 cells were culti-

Table 1. List of primers used to check cell-specific marker expression at mRNA level

Name	Sequence: (5' to 3')	Size	Tm, °C
CK10 forward primer	CTGACTGAAGAGCTGGCCTA	20	62.3
CK10 reverse primer	TTCCACATTCACATCACCAG	20	62.1
CK13 forward primer	ATGCTGCTGGACATCAAGAC	20	63.0
CK13 reverse primer	TGGTAACAGAGGTGCTACGG	20	62.7
CK14 forward primer	TGCGATCCAGAGGAGAACTG	20	65.6
CK14 reverse primer	CAGGAGATGATTGGCAGCGT	20	67.6
CK16 forward primer	GCCAATCCTATTCTTCCCGC	20	66.6
CK16 reverse primer	GGGGAGATAGCTGGGAACTG	20	64.8
CK18 forward primer	ATCTTGGTGATGCCTTGGAC	20	63.9
CK18 reverse primer	CCTGCTTCTGCTGGCTTAAT	20	63.3
CK19 forward primer	GACATGCGAAGCCAATATGA	20	63.6
CK19 reverse primer	TCAGTAACCTCGGACCTGCT	20	63.8
GAPDH forward primer	CAGCCTCCCGCTTCGCTCTC	20	72.8
GAPDH reverse primer	CCAGGCGCCCAATACGACCA	20	74.2
HPV-16 E6 forward primer	TGTTTCAGGACCCACAGGA	19	64.4
HPV-16 E6 reverse primer	TTGTTTGCAGCTCTGTGCAT	20	64.7
HPV-16 E7 forward rimer	CTGTTATGAGCAATTAATGACAGC	25	62.5
HPV-16 E7 reverse primer	CCAGCTGGACCATCTATTTC	21	63.9
Col I forward primer	AAGATGGACTCAACGGTCTC	20	60.7
Col I reverse primer	CAGGAAGCTGAAGTCGAAAC	20	61.6
Col II forward primer	ACATCACCTACCACTGCAAG	20	60.2
Col II reverse primer	TACGTGAACCTGCTATTGCC	20	62.6

Tm = Melting temperature.

vated in defined keratinocyte serum-free medium supplemented with 100 µg/ml G418. GFB-16 cells were cultivated in DF12 medium supplemented with 250 µg/ml G418.

Construction of a 3D Organotypic Gingiva Model

The method used to develop a 3D organotypic gingival model is a modification of previously reported models [Dongari-Bagtzoglou and Kashleva, 2006] with major changes described below. Briefly, the 3D organotypic gingiva models were constructed inside 13-mm-diameter Millicell cell culture inserts (Millipore) in 12-well plates. Collagen mixtures were composed of DMEM (Sigma), 10% FCS, 2 mM L-glutamine, 0.22 mg/ml sodium bicarbonate, 0.20 mM HEPES and 2.5 mg/ml rat tail Col I; 0.2 ml of this Col mixture were poured into each insert and allowed to gelify in the sterile hood to form acellular layers. Col matrix (0.5 ml) containing 7.5×10^5 GFB-16 cells were later gelled above the acellular layers. The medium used to grow fibroblasts was then added to cover each gel for incubation at 37°C in a humidified atmosphere with 5% CO₂ until the Col contracted (normally takes 1 or 2 days). The medium was then removed and the gels were plated with 1×10^6 HGEK-16 cells. The HGEK-16 cells were allowed to attach for 2 days in a mixture of HGEK-16 and GFB-16 growth medium at a ratio of 1:1 supplemented with 10 µg/ml of epithelial growth factors. Then, inserts were placed onto sterile cotton to expose the gels to the air but being still in contact with the medium. The medium used for this air-liquid interface was DMEM (4.5 mg/ml glucose) and Ham's F-12 mixed at ratio of 3:1 with 5 µg/ml insulin, 0.4 µg/ml hydrocortisone, 2×10^{-11} M 3,3',5-triiodo-L-thyronine, $1.8 \times$

10^{-4} M adenine, 5 µg/ml transferrin, 10^{-10} M cholera toxin, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 µg/ml Fungizone, 100 µg/ml G418 antibiotic and 5% FBS. Cultures were maintained in air-liquid interface for 14 days with the medium changed every 2 days.

RNA Extraction and cDNA Synthesis

For RNA extraction from monolayer cultures, cells were dissociated with 1× trypsin/EDTA and collected by centrifugation. Organotypic cultures were first washed with PBS, then resuspended in 600 µl RLT buffer (Qiagen, Basel, Switzerland) and disintegrated at 25,000 rpm in a Mini-Beadbeater-1 disrupter (BioSpec) with ice cooling between cycles and collected by centrifugation at 3,700 rpm for 1 min at 4°C. The supernatants were aliquoted into a clean tube and placed on ice for later usage. The pellets were resuspended in 590 µl RNase-free water and incubated with 20 µl proteinase K solution at 55°C for 5 min. Mixtures were then centrifuged at 10,000 rpm for 3 min at 4°C and supernatants were combined with previous ones on ice for RNA extraction. Cell lysates from all strains were kept and extracted using the RNeasy Mini Kits (Qiagen) following the instructions of the manufacturer. Clinically healthy gingiva for RNA extraction was obtained as previously described [Bostanci et al., 2009]. Briefly, gingival tissue biopsies, including both epithelium and connective tissue, were obtained from periodontally healthy subjects (n = 3) during tooth extractions for orthodontic reasons or crown-lengthening procedures. The gingival tissue biopsies were immersed in RNA stabilization reagent (RNA Later; Ambion, Foster City, Calif., USA) and

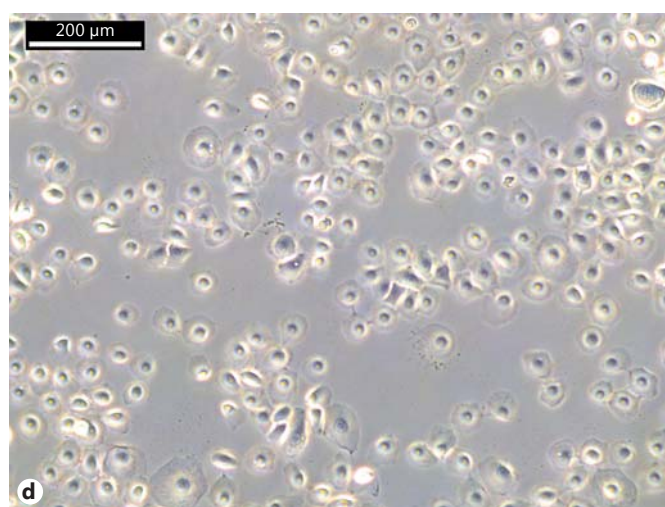
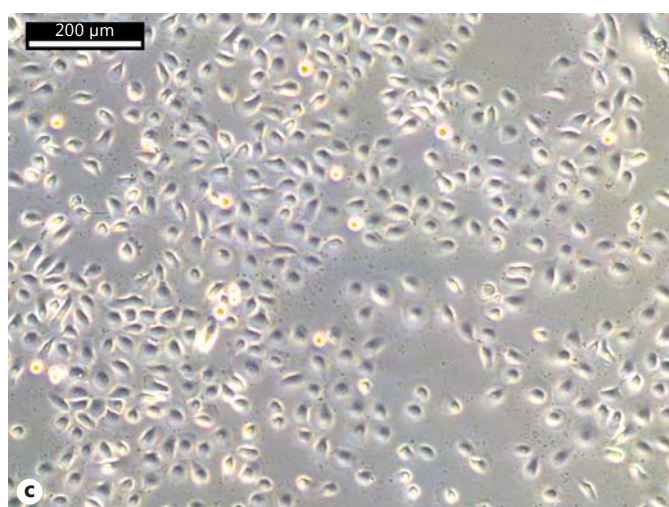
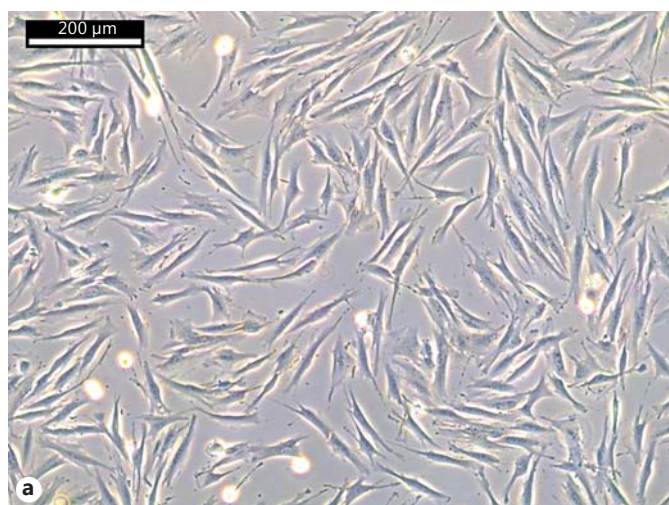
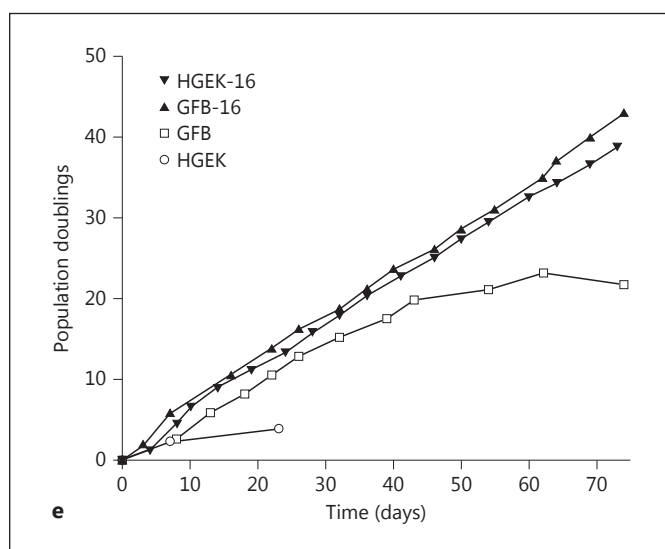


Fig. 1. Cell morphologies and growth kinetics. Phase-contrast microphotography for primary human GFB (**a**), immortalized GFB-16 (**b**), primary HGEK (**c**) and immortalized HGEK-16 (**d**) at passages 2, 13, 2 and 16, respectively. Growth kinetics of HGEK-16, GFB-16, HGEK and GFB (**e**); cells were grown in culture, and doubling times were assessed using a hemacytometer. Data are presented to day 74 until GFB cells became senescent. Each dot on the curve represents the day of subculture of the cell line. Passages 2, 6, 8 and 7 are presented for HGEK, GFB, HGEK-16 and GFB-16, respectively.



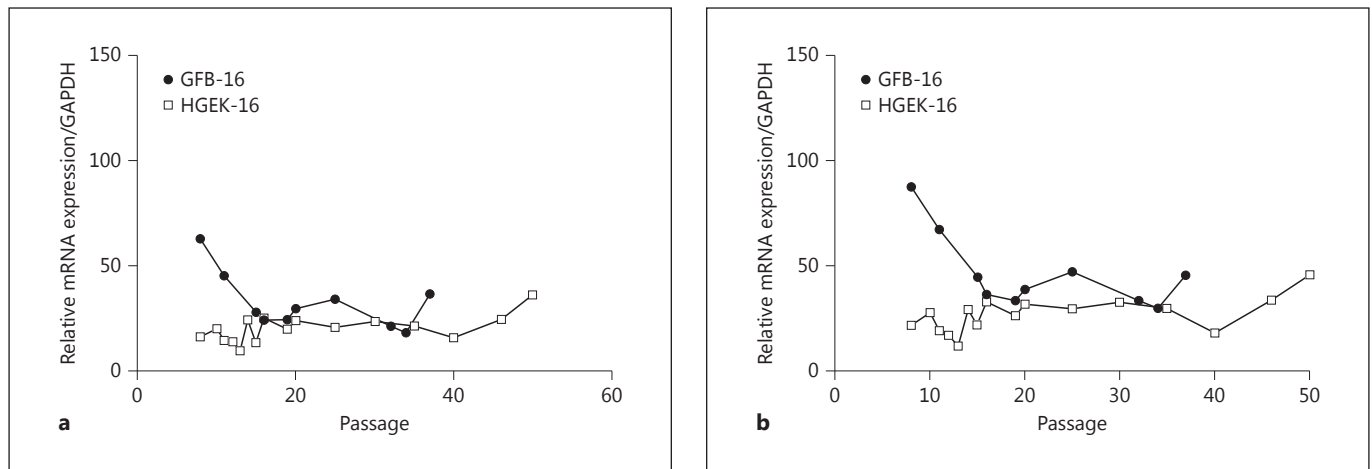


Fig. 2. HPV16-E6/E7 expression of the immortalized cell lines at different passages. mRNA expression levels of HPV16-E6 (**a**) and -E7 (**b**) were normalized with a housekeeping gene (GAPDH).

then frozen at -80°C . Total RNA was extracted using the AllPrep Kit (Qiagen). Moloney murine leukemia virus reverse transcriptase (Promega) was used to synthesize cDNA according to the manufacturer's instruction.

qRT-PCR

For the characterization of gene expression in both 2D and 3D cultures, CK10, CK13, CK14, CK16, CK18, CK19, Col I and Col II were amplified using StepOnePlus™ real-time PCR systems (Applied Biosystems) following the standard instructions of the manufacturer and data were analyzed by the software provided. Oligonucleotide primer sequences are listed in table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous RNA control in the samples (housekeeping gene). The SYBR Green PCR Master Mix (Applied Biosystems) was used for amplification reactions.

3D Model Treatments for Immunohistology

Samples were rinsed twice with PBS for 5 min and then transferred into 3% paraformaldehyde supplemented with 2% sucrose/PBS fixation medium for 2 h. The samples were rinsed thrice with PBS, 20 min each; dehydrated by immersion in 15% sucrose/PBS for 2 h at room temperature, and then left in 30% sucrose/PBS overnight at 4°C . The next day, samples were placed in 5 ml of 5% (w/w) porcine-derived gelatin (Sigma) solution supplemented with 5% (w/w) sucrose that was prewarmed to 45°C in the models. The whole models were transferred in a cool 2-methylbutane (isopentane) and dry ice bath at around -40°C until they were frozen. Specimens were stored at -80°C and sectioned at 8- μm thickness using a HYRAX C 50 cryostat (Zeiss, Jena, Germany).

Histological Staining and Image Composition

Cryosection slides at -80°C were dried at room temperature for 0.5–2 h and then fixed in acetone (4°C) for 15 min. After washing thrice with PBS, the specimens were blocked with 1% BSA/PBS for 10 min. The specimens were then incubated with 4 $\mu\text{g}/\text{ml}$ mouse monoclonal keratin 14 Ab-1 (Clone LL002; Neomarker) or

4 $\mu\text{g}/\text{ml}$ keratin 16 Ab-1 (Clone LL025; Neomarker) at 4°C overnight. After removal of the primary antibody, the Alexa Fluor® 488 goat anti-mouse IgG (H+L) antibody was applied to the samples and incubated for 45 min at room temperature. DAPI was later applied on the samples as a counterstain.

Statistical Evaluation

The results of qRT-PCR analysis for the expression levels of different transcripts in each sample were calculated by the comparative Ct method ($2^{-\Delta\text{Ct}}$ formula) after normalization to endogenous control GAPDH (i. e. $\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$). All data are expressed as means \pm SD for all of the determinations. Significance was calculated from log values of ΔCt between different conditions. Unpaired t tests were used to test the differences between immortalized cell lines with their parent primary cells. Multiple comparison tests following one-way ANOVA were used to test the differences between 3D organotypic gingiva models, clinical samples and 2D monolayer cultures.

Results

Characterization of Immortalized Human Gingival Epithelial and Fibroblastic Cell Lines in 2D Monolayer Cultures

Immortalized Cell Lines Show Cell Morphologies and Growth Kinetics Similar to Their Parental Cells in Monolayer Cultures

Primary HGEK and GFB exhibited distinct morphologies under the light microscope (fig. 1). Keratinocytes exhibited characteristic epithelial cobblestone morphologies (fig. 1c, d), while fibroblasts exhibited typical spindle-shaped fibroblast morphologies (fig. 1a, b). HPV16 immortalized clones of both keratinocytes and fibroblasts

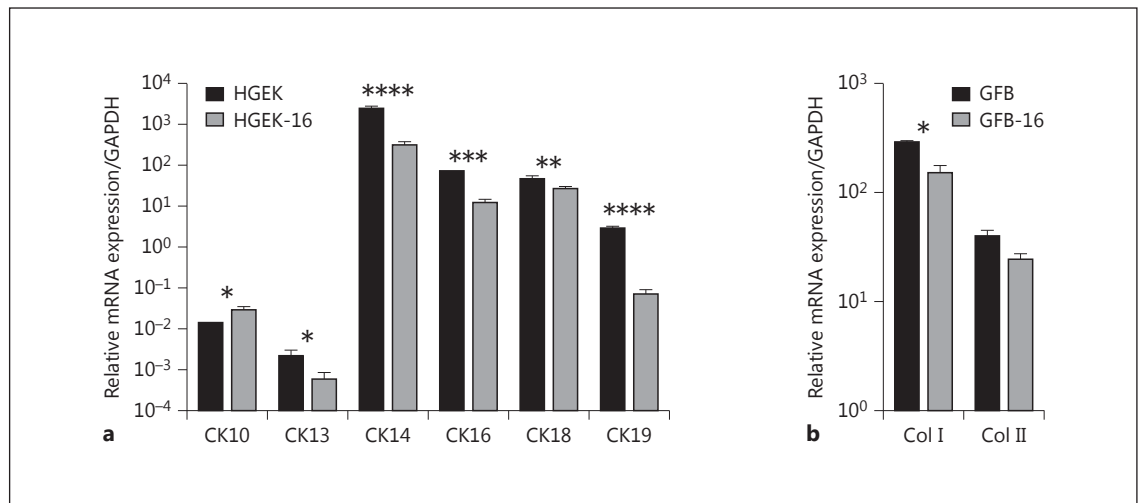


Fig. 3. Expression of differentiation markers in primary and immortalized cells from different passages. **a** The values of CK10, CK13, CK14, CK16, CK18 and CK19 represent values of 3 or 18 passages, respectively, for primary (HGEK) or immortalized epithelial cells (HGEK-16; means \pm SD) as described in Materials and Methods. **b** The expression levels of Col I and II were compared between primary (GFB) and immortalized cells (GFB-16). Values (means \pm SD) of 4 and 10 passages for GFB and GFB-16,

respectively (online suppl. table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000363694) as described in Materials and Methods. For a better understanding of the data, results are presented on a logarithmic scale. For each marker, mRNA levels were normalized with a housekeeping gene (GAPDH). * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.00001$, primary cells vs. immortalized cells.

showed morphologies similar to their parental cells at early passages, although primary cells were more heterogeneous in their morphology.

The subculture time of primary gingival keratinocytes and fibroblasts remained similar in early passages, but cells proliferated more slowly and became senescent at 4 and 18 population doublings, respectively. In comparison, immortalized cells showed continuous proliferation and expansion over 30 population doublings whilst retaining a similar cobblestone or spindle-shape phenotype. The growth behavior of the immortalized cell lines remained constant over time and required subcultivation every 4–5 days (fig. 1e).

Immortalized Cell Lines Express Stable Levels of HPV16-E6/E7

To test for the presence and expression levels of the HPV16-E6/E7, mRNAs of HGEK-16 and GFB-16 were extracted from the cells in different passages and examined by qRT-PCR (fig. 2). The analysis confirmed oncogene expression in all HPV16-E6/E7-transduced cells at all detected passages at a similar level. No oncogene expression was detected in the corresponding parental primary cells (data not shown).

Immortalized Cell Lines in Monolayer Cultures Express Similar Levels of Cell-Specific Markers when Compared with Their Parental Cells

Since CK are the main intermediate filaments of gingival epithelia, mRNA levels of selected CK were examined as differentiation markers. Therefore, CK10, CK13, CK14, CK16, CK18 and CK19 were tested in primary HGEK and HGEK-16 cells by qRT-PCR (fig. 3a). Essentially, HGEK and HGEK-16 cells all expressed these CK, although slight effects on CK expression by HPV16-E6/E7 were noted. Both cell types expressed low levels of CK10 and CK13 and high levels of CK14, CK16, CK18 and CK19 (fig. 3a). Immunohistochemical analysis also confirmed strong expression of CK14 in HGEK-16 monolayer cultures (fig. 4c). As a marker of cornifying epithelium, CK10 was expressed at low level when compared to CK14, the marker of stratifying epithelium, which was expressed at highest levels. The marker of non-cornified epithelium, CK13, was present but at lower levels. Magnitudes of gene expression of the selected CK were compared between HGEK and HGEK-16. CK10 and CK13 were expressed at slightly higher levels in HGEK-16 (0.45-fold, $p = 0.0237$, and 3.60-fold, $p = 0.0399$, respectively) compared with HGEK. In contrast, HGEK-16 expressed lower levels of CK14, CK16, CK18 and CK19 (7.44-fold,

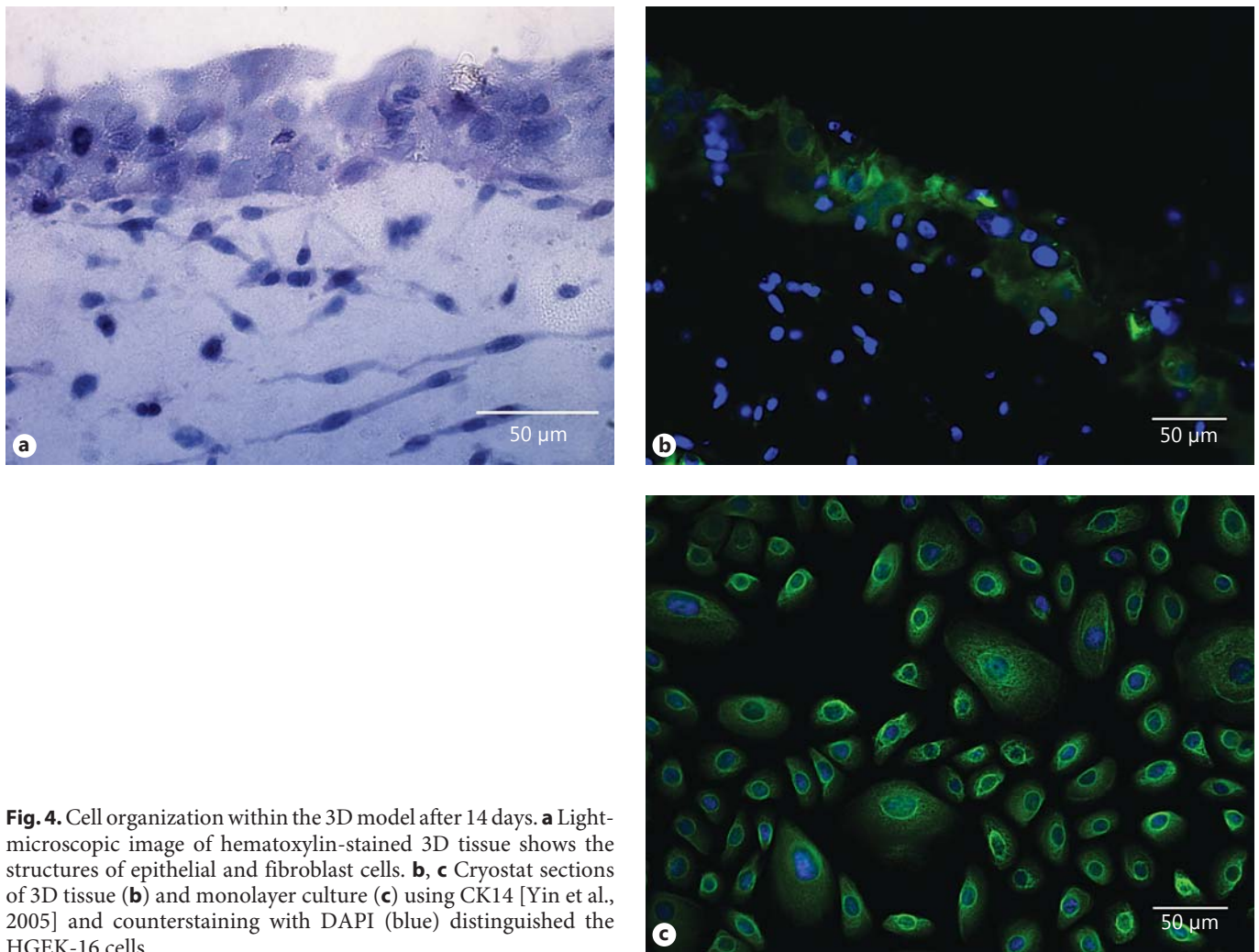


Fig. 4. Cell organization within the 3D model after 14 days. **a** Light-microscopic image of hematoxylin-stained 3D tissue shows the structures of epithelial and fibroblast cells. **b, c** Cryostat sections of 3D tissue (**b**) and monolayer culture (**c**) using CK14 [Yin et al., 2005] and counterstaining with DAPI (blue) distinguished the HGEK-16 cells.

$p < 0.00001$; 5.62-fold, $p = 0.0002$; 1.58-fold, $p = 0.0087$, and 40.13-fold, $p < 0.00001$, respectively) compared with HGEK.

In addition, expression levels of extracellular matrix-related genes (i.e. Col I and Col II) were examined for GFB and GFB-16. Both fibroblast cell types demonstrated strong expression of Col I and Col II (fig. 3b), with Col I expression in GFB-16 being slightly lower (1.62-fold, $p = 0.024$) than in GFB.

Behavior of Immortalized Human Gingival Epithelial and Fibroblastic Cells in 3D Organotypic Cultures

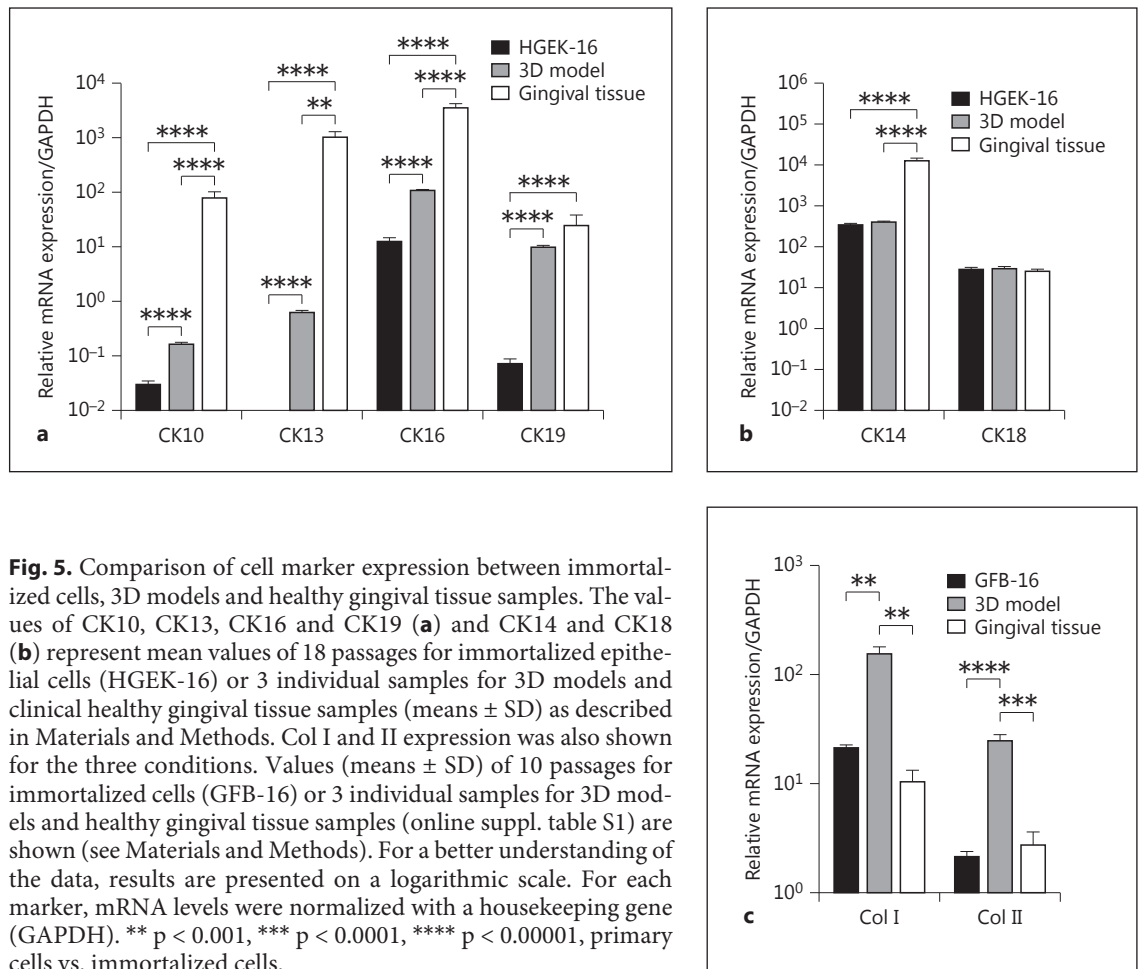
3D Organotypic Cell Cultures Showed a Multilayer Structure

GFB-16 and HGEK-16 cells were grown to confluence in monolayer cultures and then used to construct 3D or-

ganotypic cultures. Figure 4a shows a representative section of this model stained with hematoxylin. HGEK-16 cells formed a multilayered regenerated epithelium with no obvious keratinizing superficial layer on the surface, which is similar to the non-keratinized oral epithelia such as junctional epithelium in vivo. The HGEK-16 cells formed multilayers of epithelia and GFB-16 fibroblasts were evenly distributed in the Col gel matrix. Like in monolayer cultures (fig. 4c), HGEK-16 epithelium in 3D organotypic cultures revealed strong CK14 staining (fig. 4b).

Gene Expression Levels of CK and Col in 3D Organotypic Cultures Were Closer to the Levels in Healthy Gingiva than in 2D Monolayer Cultures

One of the key differences between in vitro and in vivo epithelia is that they express epithelial markers different-



ly [Locke et al., 2008]. We expected the CK expression levels in organotypic cultures to be closer to the levels found in vivo in human tissue than in monolayer culture. Therefore, CK expression in organotypic cultures, healthy gingival tissue and monolayer cultures of HGEK-16 were compared using qRT-PCR. In addition, mRNA expression levels of Col I and Col II were compared between organotypic cultures, clinical samples and GFB-16 cells grown as monolayers.

qRT-PCR analysis showed that the expression levels of CK in HGEK-16 organotypic cultures are closer to those in gingival tissue than to monolayer cell cultures (fig. 5a, b). Especially for CK19, 3D model expression is 135.91-fold higher than in the immortalized cells, with no significant difference to the level in gingival tissue models (2.50-fold, $p > 0.05$). The levels of CK10, CK13 and CK16 in gingival tissue were significantly higher (465.21-fold, $p < 0.00001$; 1,658.49-fold, $p < 0.0001$, and 32.07-fold,

$p < 0.00001$, respectively) than the levels in the 3D models. However, these values are significantly less than the difference between gingival tissue (2,674.31-fold, $p < 0.00001$; 1,785,091.51-fold, $p < 0.00001$, and 287.61-fold, $p < 0.00001$, for CK10, CK13 and CK16, respectively) and HGEK-16 grown as monolayers.

CK14 and CK18 expression in 3D models showed no significant differences with monolayer parallel cultures (fig. 5b). Both 3D models and HGEK-16 monolayers expressed significantly lower levels of CK14 compared with gingival tissue (0.027-fold, $p < 0.00001$, and 0.032-fold, $p < 0.00001$, for HGEK-16 and 3D models compared with gingival tissue, respectively), while for CK18, HGEK-16 monolayer cells, 3D cultures and gingival tissue showed no significant difference in expression. Col I and Col II expression was found to be significantly higher in GFB-16 monolayer (14.49-fold, $p < 0.000$, and 8.29-fold, $p < 0.00001$, respectively) than the levels in gingival tissue

(fig. 5c). However, expression of both Col genes showed no significant differences between gingival tissue and the 3D model.

Discussion

To decipher the pathologies of periodontal diseases, it is important to establish *in vitro* models that mimic the mechanisms taking place within the gingival tissue. In the present study, we established epithelial and fibroblastic cell lines from human gingiva. Both epithelial and fibroblastic cell lines demonstrated a stable infinite growth with epithelium-like and spindle fibroblastic-shape morphology, respectively. Additionally, 3D multilayered organotypic culture was formed by use of embedded fibroblasts in a Col gel.

It has long been known that connective tissue underlying epithelia not only supports growth but also regulates differentiation of the epithelia [Karring et al., 1975]. Therefore, such organotypic cultures should consist of both epithelium and connective tissue. For the purpose of generating such a model, we developed and characterized immortalized cell lines (HGEK-16 and GFB-16) through the expression of HPV16-E6/E7 oncogenes. Stable expression levels of E6 and E7 throughout many passages confirmed successful immortalization. In our study, no morphology difference was observed between primary cultures and immortalized cell lines even after 50 (HGEK-16) and 35 passages (GFB-16). Earlier studies have reported immortalized human gingival fibroblasts by gene transfection of human telomerase reverse transcriptase [Kamata et al., 2004; Illeperuma et al., 2012], although other studies have shown that co-expression of human telomerase reverse transcriptase with other immortalizing genes, i.e. HPV16-E6/E7, was required for efficient immortalization of oral epithelial cells [Kamata et al., 2004].

The HGEK-16 cells had characteristic ‘cobblestone’ appearance of oral epithelial cells, while GFB-16 cells exhibited spindle-shaped morphology as their primary counterparts. qRT-PCR analysis showed that, like primary gingival fibroblasts, GFB-16 cells express highly abundant levels of Col I. Regarding the expression of Col II in the present experimental system, while its expression is not a typical feature of the gingival mucosa, there is evidence that under certain conditions GFB can express this and have potential to differentiate into chondrocyte-like cells [Yeh et al., 2013].

During periodontal infection, the junctional epithelium is exposed to a biofilm, and this tissue is therefore the subject of intense study into this disease [Bosshardt and Lang, 2005]. Several earlier studies have reported that gingival epithelial cells can form a multilayered epithelium when co-cultured with fibroblastic cells within 3D matrices allowing more direct comparisons to *in vivo* models [Dongari-Bagtzoglou and Kashleva, 2006; Roesch-Ely et al., 2006]. Indeed, when the transformed HGEK cells were co-cultured in a Col matrix along with human gingival fibroblasts, their characteristics were qualitatively and quantitatively closer to those in human gingival tissue than to monolayer cultures. One of the drawbacks of utilizing immortalized cell lines in organotypic cultures is that these cells may show carcinoma-like invasion patterns into the underlying connective tissue, especially in the presence of fibroblast/Col matrices [Costea et al., 2006]. Fortunately, in our model, HGEK-16 showed no invasive trend into the fibroblast/Col matrix when both cell lines were used to concomitantly construct organotypic tissue.

Within the gingiva, the expression patterns of various CK have been used as molecular indicators for different oral gingival epithelium regions. Previous studies showed varying expression levels between the keratinized region, the non-keratinized oral junctional epithelium and sulcular epithelium [Bragulla and Homberger, 2009; Hsieh et al., 2010]. Therefore, CK10, CK13, CK14, CK16, CK18 and CK19 were chosen as cell-specific markers [Locke et al., 2008]. CK19 is considered a marker for early differentiation stages of stratified epithelia [Groger et al., 2008] and is mostly found in simple epithelia, such as the junctional epithelium [Mackenzie and Gao, 1993]. Therefore, increased expression of CK19 in the present 3D organotypic tissue model is a common feature of junctional epithelium. CK10 and CK16 are known to be largely expressed in cornifying stratified epithelia [Coulombe and Omary, 2002] as well as in proliferating epithelia [Freedberg et al., 2001]. Thus, healthy gingival tissue samples, which contain mainly keratinized epithelia, expressed very high levels of these keratins. In contrast, the expression of these two CK was significantly lower in the present organotypic tissue compared to patient tissue. As these are considered essential for terminal differentiation of keratinized epithelia, low expression of CK10 and CK16 in our model confirms that the formed multilayered epithelium is rather non-keratinized.

In conclusion, the immortalized cell lines generally inherited the characteristics of their parental primary tissue cells. When cultured in 3D Col matrix, these immortal-

ized cell lines resembled their in vivo counterpart more closely than the corresponding monolayer cultures. These results indicate that our new cell lines may be useful tools for studying the physiology and pathobiology of gingival tissue in vitro. Moreover, this could present a practical model for studies of biofilm-gingival tissue interactions in vitro [Belibasakis et al., 2014; Belibasakis and Thurnheer, 2014]. The development of this model is not necessarily restricted to the gingiva, but could potentially be

expanded with adequate modifications for applications on the respiratory or gastrointestinal tract mucosa, or the skin.

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References

- Akgul, B., N. Bostanci, K. Westphal, I. Nindl, H. Navsaria, A. Storey, H. Pfister (2010) Human papillomavirus 5 and 8 E6 downregulate interleukin-8 secretion in primary human keratinocytes. *J Gen Virol* 91(Pt 4): 888–892.
- Albandar, J.M. (2011) Underestimation of periodontitis in NHANES surveys. *J Periodontol* 82: 337–341.
- Belibasakis, G.N., A. Johansson, Y. Wang, C. Chen, S. Kalfas, U.H. Lerner (2005) The cytolethal distending toxin induces receptor activator of NF- κ B ligand expression in human gingival fibroblasts and periodontal ligament cells. *Infect Immun* 73: 342–351.
- Belibasakis, G.N., A. Meier, B. Guggenheim, N. Bostanci (2011) The RANKL-OPG system is differentially regulated by supragingival and subgingival biofilm supernatants. *Cytokine* 55: 98–103.
- Belibasakis, G.N., K. Bao, N. Bostanci (2014) Transcriptional profiling of human gingival fibroblasts in response to multi-species in vitro subgingival biofilms. *Mol Oral Microbiol* 29: 174–183.
- Belibasakis, G.N., T. Thurnheer (2014) Validation of antibiotic efficacy on in vitro subgingival biofilms. *J Periodontol* 85: 343–348.
- Bosshardt, D.D., N.P. Lang (2005) The junctional epithelium: from health to disease. *J Dent Res* 84: 9–20.
- Bostanci, N., G. Emingil, B. Saygan, O. Turkoglu, G. Atilla, M.A. Curtis, G.N. Belibasakis (2009) Expression and regulation of the NALP3 inflammasome complex in periodontal diseases. *Clin Exp Immunol* 157: 415–422.
- Bragulla, H.H., D.G. Homberger (2009) Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J Anat* 214: 516–559.
- Costea, D.E., K. Kulasekara, E. Neppelberg, A.C. Johannessen, O.K. Vintermyr (2006) Species-specific fibroblasts required for triggering invasiveness of partially transformed oral keratinocytes. *Am J Pathol* 168: 1889–1897.
- Coulombe, P.A., M.B. Omary (2002) 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr Opin Cell Biol* 14: 110–122.
- Dongari-Bagtzoglou, A., H. Kashleva (2006) Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. *Nat Protoc* 1: 2012–2018.
- Freedberg, I.M., M. Tomic-Canic, M. Komine, M. Blumenberg (2001) Keratins and the keratinocyte activation cycle. *J Invest Dermatol* 116: 633–640.
- Groger, S., J. Michel, J. Meyle (2008) Establishment and characterization of immortalized human gingival keratinocyte cell lines. *J Periodontol Res* 43: 604–614.
- Guggenheim, B., R. Gmür, J.C. Galicia, P.G. Stathopoulou, M.R. Benakanakere, A. Meier, T. Thurnheer, D.F. Kinane (2009) In vitro modeling of host-parasite interactions: the 'subgingival' biofilm challenge of primary human epithelial cells. *BMC Microbiol* 9: 280.
- Hsieh, P.C., Y.T. Jin, C.W. Chang, C.C. Huang, S.C. Liao, K. Yuan (2010) Elastin in oral connective tissue modulates the keratinization of overlying epithelium. *J Clin Periodontol* 37: 705–711.
- Illeperuma, R.P., Y.J. Park, J.M. Kim, J.Y. Bae, Z.M. Che, H.K. Son, M.R. Han, K.M. Kim, J. Kim (2012) Immortalized gingival fibroblasts as a cytotoxicity test model for dental materials. *J Mater Sci Mater Med* 23: 753–762.
- Kamata, N., R. Fujimoto, M. Tomonari, M. Taki, M. Nagayama, S. Yasumoto (2004) Immortalization of human dental papilla, dental pulp, periodontal ligament cells and gingival fibroblasts by telomerase reverse transcriptase. *J Oral Pathol Med* 33: 417–423.
- Karring, T., N.P. Lang, H. Loe (1975) The role of gingival connective tissue in determining epithelial differentiation. *J Periodontol Res* 10: 1–11.
- Locke, M., P.L. Hyland, C.R. Irwin, I.C. Mackenzie (2008) Modulation of gingival epithelial phenotypes by interactions with regionally defined populations of fibroblasts. *J Periodontol Res* 43: 279–289.
- Mackenzie, I.C., Z. Gao (1993) Patterns of cytokeratin expression in the epithelia of inflamed human gingiva and periodontal pockets. *J Periodontol Res* 28: 49–59.
- MacNeil, S. (2007) Progress and opportunities for tissue-engineered skin. *Nature* 445: 874–880.
- Nanci, A., D.D. Bosshardt (2006) Structure of periodontal tissues in health and disease. *Periodontol* 2000 40: 11–28.
- Pi, S.H., S.K. Lee, Y.S. Hwang, M.G. Choi, S.K. Lee, E.C. Kim (2007) Differential expression of periodontal ligament-specific markers and osteogenic differentiation in human papilloma virus 16-immortalized human gingival fibroblasts and periodontal ligament cells. *J Periodontol Res* 42: 104–113.
- Radyuk, S.N., P.A. Mericko, T.G. Popova, E. Grene, K. Alibek (2003) In vitro-generated respiratory mucosa: a new tool to study inhalational anthrax. *Biochem Biophys Res Commun* 305: 624–632.
- Roesch-Ely, M., T. Steinberg, F.X. Bosch, E. Musig, N. Whitaker, T. Wiest, A. Kohl, G. Komposch, P. Tomakidi (2006) Organotypic cocultures allow for immortalized human gingival keratinocytes to reconstitute a gingival epithelial phenotype in vitro. *Differentiation* 74: 622–637.
- Sacks, P.G. (1996) Cell, tissue and organ culture as in vitro models to study the biology of squamous cell carcinomas of the head and neck. *Cancer Metastasis Rev* 15: 27–51.
- Sandros, J., P.N. Papapanou, U. Nannmark, G. Dahlen (1994) *Porphyromonas gingivalis* invades human pocket epithelium in vitro. *J Periodontol Res* 29: 62–69.
- Smola, H., H.J. Stark, G. Thiekötter, N. Miranica, T. Krieg, N.E. Fusenig (1998) Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. *Exp Cell Res* 239: 399–410.
- Yeh, H.Y., T.Y. Lin, C.H. Lin, B.L. Yen, C.L. Tsai, S.H. Hsu (2013) Neocartilage formation from mesenchymal stem cells grown in type II collagen-hyaluronan composite scaffolds. *Differentiation* 86: 171–183.
- Yin, T., S. Getsios, R. Caldelari, L.M. Godsel, A.P. Kowalczyk, E.J. Muller, K.J. Green (2005) Mechanisms of plakoglobin-dependent adhesion: desmosome-specific functions in assembly and regulation by epidermal growth factor receptor. *J Biol Chem* 280: 40355–40363.